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PRINCIPAL INVESTIGATOR: Michael A. Schwarzschild, M.D., Ph.D.

CONTRACTING ORGANIZATION:
MASSACHUSETTS GENERAL HOSPITAL
RESEARCH MANAGEMENT
50 STANIFORD STREET, SUITE 1001
BOSTON MA 02114-2554

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6. AUTHOR(S)
Michael A. Schwarzschild, M.D., Ph.D. (PI)
Kui Xu, M.D., PhD. (co-investigator)
Email: michaels@helix.mgh.harvard.edu

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Research Management
50 Staniford Street, Suite 1001
Boston, MA 02114-2554

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
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14. ABSTRACT

Continued progress has been made toward each of the Specific Aims (SAs) 1 and 2 (SA 3 completed) of our research project, "Caffeine, adenosine receptors and estrogen in toxin models of Parkinson's disease (PD)". The overarching hypothesis of the project is that *multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in PD*. To that end we are characterizing the interplay between several environmental agents (pesticides, caffeine and estrogen) that are leading candidate modulators of PD risk. In Year 4 we have obtained and reported evidence that the adenosine receptor blocker caffeine as well as specific genetic depletion of the A_{2A} subtype of adenosine receptor are capable of conferring neuroprotection in chronic pesticide and genetic mouse models of PD. Critical to our ability to successfully pursue the Specific Aims of our research program, we have made technical progress in assessing modification of a key conditioanl KO methodology developed in this project. We have presented initial data applying this method to the MPTP model of neurodegeneration in PD.

15. SUBJECT TERMS
adenosine, caffeine, estrogen, neuroprotection, neurotoxin, Parkinson's disease

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Introduction

Identifying the mechanisms by which caffeine and more specific A_{2A} antagonists protect dopaminergic neurons in multiple models of Parkinson's disease (PD) will advance our knowledge of the pathophysiology, epidemiology and therapeutics of PD.

The *overarching hypothesis* pursued by this proposal is that **multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease**. Here we endeavor to characterize the interplay between several environmental agents (pesticides, caffeine, estrogen and most recently the caffeine-related purine urate) that are leading candidate modulators of PD risk.
We are pursuing 3 *specific hypotheses:*

1) Caffeine acts through blockade of brain A_{2A}Rs to protect dopaminergic neurons in both acute and chronic toxin models of PD. (***Specific Aim #1***)

2) Caffeine perfusion and focal A_{2A}R inactivation within the striatum are sufficient to attenuate MPTP toxicity, by reducing toxin-induced release of glutamate and/or GABA. (***Specific Aim #2***)

3) Estrogen attenuates the protective effect of caffeine but not the protective of A_{2A}R deletion because it acts by altering caffeine metabolism or A_{2A}R expression. (***Specific Aim #3***)

Statement of Relevance (from our proposal)

A. Parkinson's Disease -

Basic neuroscience significance - The results will improve our understanding of adenosine receptor neurobiology, and will provide insight into the role of endogenous adenosine in basal ganglia biology physiology and PD pathophysiology.

Epidemiological significance - Establishing the ability of caffeine to protect dopaminergic neurons in PD models and identifying a plausible mechanism of action greatly strengthens the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with risk of PD.

Therapeutic significance - With several specific adenosine A_{2A} antagonists emerging as promising therapeutic candidates based on their motor-enhancing (symptom-relieving) action, the prospects for additional neuroprotective benefit substantiated by this project may considerably enhance their therapeutic potential. In addition, identifying a biological basis for caffeine-estrogen interaction in modifying PD risk could also affect recommendations for estrogen replacement strategies in women with PD taking A_{2A} antagonists or caffeine (and *vice versa*). Furthermore, based on evidence that A_{2A}Rs contribute to the neurotoxicity affecting cortical and striatal neurons (as well as dopaminergic neurons), our findings may support novel A_{2A}R-

based neuroprotective treatments for a wider range of neurological diseases from stroke to amyotrophic lateral sclerosis (ALS) to Alzheimer's disease.

B. Environmental Neurotoxin Exposure in Military Service – By characterizing the neuroprotective effects of caffeine in a chronic pesticide model of PD (as well as the acute MPTP model), the proposed work will define a prototypical interaction between environmental toxins and protectants in determining the extent of a well-characterized neurological lesion (dopaminergic neuron death). Although there has been no compelling evidence to suggest that the incidence of PD will itself increase in association with military service or combat theatre exposures,[1] putative toxin exposure in the military may be linked to the development of another debilitating neurodegenerative disorder, ALS.[2] Moreover, some objective biological measures in veterans diagnosed with a "Persian Gulf War syndrome" have indicated dysfunction of dopaminergic neurotransmission in the basal ganglia,[3] raising the possibility (together with other data[4]) of altered risk for PD in this group. In any event, establishing a biological precedent for neurotoxin-neuroprotectant interplay in the relatively common disorder of PD, may provide a 'roadmap' that can be used should any neurological illness be confirmed to develop in association with prior military exposures.

C. Understanding the Non-stimulant CNS Effects of Caffeine. The psychoactive agent caffeine has been endorsed for military use at relatively high doses to help maintain operational readiness.[5] This recommendation has been based on a large body of evidence demonstrating sustainment of mental task performance by caffeine, and a lack of evidence for substantial harm at these doses. However, adopting the use of any CNS-active drug by protocol warrants careful consideration of newly appreciated neuronal actions of the agent. Accordingly, the proposed investigation of the novel neuroprotective effect of caffeine and its underlying mechanisms (e.g., altered neurotransmitter release) would be of significance for military programs that provide specific doses of caffeine to personnel to enhance cognitive function.

D. Gender Differences in How Environmental Factors Impact Toxin Susceptibility. Our investigation of how caffeine and estrogen exposures interact to modify neurotoxin susceptibility in laboratory models of PD may have substantial significance for the human epidemiology that prompted our pursuit of this line of research. In addition, the proposed studies may provide a prototype for modeling how gender and estrogen status interact with environmental exposures of relevance to the military (i.e., neurotoxins, caffeine). A better appreciation of how gender alters susceptibility to environmental toxins or protectants may ultimately lead to a better understanding (and modification) of the differential risks faced by women and men serving in the same military operations.

Body of the Report

Progress during Year 4 on Specific Aims and experiments as laid out in our original Statement of Work (SOW [in blue]) is described here in detail.

STATEMENT OF WORK (focus on main areas of progress in Yr 4)

Specific Aim #1 – to definitively determine whether brain A_{2A}Rs or A_1Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain A_{2A}R is required for caffeine's protective effect in these PD models.

[Please see manuscript and abstract publication in Appendices A and B.]

- Completion of work demonstrating that the non-specific adenosine antagonist caffeine can in fact confer neuroprotection in a chonic dual pesticide toxin model of PD. Pretreatment with caffeine attenuated loss of nigral dopaminergic neurons in mice chronically exposed (twice weekly for two months) to paraquat plus maneb. Rigorous stereological assessments were made of tyrosine hydroxylase-immunoreactive cells to determine total dopaminergic neuron counts of the substantia nigra. A manuscript reporting these findings has now been submitted for publications (Appendix A).
- In Year 4 we have also investigated and preliminarily reported (Appendix B) how depletion of adenonsine A_{2A} receptors (in A_{2A} knockout mice) may mimic the neuroportective effect of caffeine in a chronic mouse model of PD. Here we used an lifelong exposure to an 'endogenous toxin' of transgenically expressed mutant human α–synuclein (*hw-αSYN*). Mutations in the human α–synuclein gene (A53T and A30P) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human α–synuclein gene with both of these mutations have been shown to develop age-related reductions in dopaminergic nigral neuron numbers, striatal dopamine (DA) and motor activity.

  We investigated whether deletion of the A_{2A} receptor (in A_{2A}[-/-] mice) would afford protection against the age-related spontaneous reduction of striatal dopamine in the double-mutant human α-synuclein (*hm²-αSYN*) mice. Heterozygous A_{2A}[+/-] mice were mated with A_{2A}[+/-] mice that were also transgenic for the wild-type (*hw-αSYN*) or *hm²-αSYN* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter – all in a congenic C57Bl/6 background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional A_{2A} receptor, DA content was significantly lower (by 35%) in hm²-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in A_{2A}[-/-] mice (lacking functional A_{2A} receptor) the hm²-αSYN transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender. Stereological nigral TH-IR cell counts are in progress. Thus the A_{2A} receptor appears to be required for neurotoxicity in an α–synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific A_{2A} antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

Specific Aim #2 – to localize the region within brain through which caffeine or A_{2A} receptor inactivation produces its neuroprotective effect in the MPTP model of PD.

[Please see abstract publication in Appendix C.]

Hypothesis 2: **Caffeine perfusion and focal A_{2A} receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

Exp# 5 – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed A_{2A}R mice**: Homozygous floxed A_{2A}R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP...

Progress in Year 4 has been focused on working through technical limitations of potential AAV toxicity confounding interpretation of the hypothesized protective effect conferred by the Cre-medicated recombination event produced by viral expression of *cre* in the striatum. As reported in Year 3 (see Progress Report Yr 3), using the AAV-Cre/*lox*P system, with a double injection of AAV2/1-*cre* ($3x10^{13}$ gc/ml), in the rostral and caudal striatum respectively, we demonstrated the induction of a 50% loss of A_{2A} striatal receptors in $A_{2A}^{flox/flox}$ mice. This focal A_{2A}R KO was still stable until 60 days post injection and was sufficient to induce a behavioral phenotype and to confer partial but significant neuroprotection against acute MPTP intoxication (see Appendix C). On the other hand at that time (60 days post injection) cell toxicity was detected in the center of the infected area in some mice, probably related to an over-expression of Cre recombinase across time. To reduce this late cell toxicity in collaboration with Dr. Miguel Sena-Esteves at our institution, we tested a promising alternative AVV vector, which delivers the Cre recombinase under control of a weaker promoter: the rat synapsin 1 promoter, that should confer a higher neuronal specificity for the infection and reduce the amount of Cre expressed in every single cell (and, we reasoned, should reduce the toxicity accordingly).

Although AAV2/1-syn1-*cre* ($1x10^{13}$ gc/ml and $6x10^{13}$ gc/ml) injection in the striatum of $A_{2A}R^{flox/flox}$ mice did not induce a Cre expression detectable with IHC techniques (as we expected because of the weaker promoter), it did not induce appreciable loss of A_{2A}R-IR loss in the brain tissue surrounding the injection area. Nevertheless, a further analysis of the targeted neurons revealed also a loss of DARPP-32-IR (a marker for srtiatal neurons, which express high levels of this protein) in the same area. Thus, it appears attempt to obviate this technical limitation was not successful, and we have returned to the successful original promoter AAV construct and will pursue alternative strategies to minimize it's long-term toxicity.

Detailed method: Mice with a *floxed* $A_{2A}R$ gene were generated by insertion of *lox*P sequences within the introns flanking the exon 2 of the $A_{2A}R$ gene (YJ Day and J linden, unpublished results). Homozygous adult male mice were anesthetized with an i.p. injection of Avertin and positioned in a stereotaxic frame for double injection into the left striatum (rostral: AP: +1.2; ML: +1.5; DV: -2.75; caudal: AP: -0.15; ML: +2.5; DV: -2.65) of 1 μl of AAV2/1syn1-*cre* ($1x10^{13}$ and $6x10^{13}$ gc/ml) at a rate of 0.1 μl/min by using a 30 gauge needle connected to a 50μl Hamilton syringe driven by a microinfusion pump.

After 16, 30 and 60 mice were anesthetized and intracardially perfused with 10 ml of ice-cold saline followed by 30 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer. After perfusion brains were removed and incubated overnight in the same fixative than cryoprotected by incubation in phosphate buffered 30% sucrose. Serial 35 μm-thick coronal sections were cut on a freezing microtome and collected in 50 mM Tris buffer. Adjacent sections starting from the rostral part of the Striatum to the Sunstantia Nigra pars compacta (Snc) were collected for immunohistochemical staining for Cre recombinase (1:2000 Novagen anti-Cre rabbit polyclonal antibody) $A_{2A}R$ (1:200 Santa Cruz anti $A_{2A}R$ goat polyclonal antibody) and DARPP-32 (1:1000 Cell Signaling anti-DARPP-32 rabbit polyclonal antibody). Goat or Horse anti-species antibodies conjugated to biotin, Vectastain ABC Kit and fast DAB Kit were used for detecting primary antibodies. Immunostainig controls were done without the primary antibody. Quantification of Cre, A2aR and DARPP32-immunoreactivity (IR) was performed every sixth section at 4X magnification by measuring the extent of the immunopositive or immunonegative area in the striatum.

Key Research Accomplishments (in Year 4)

- Completion of work and submission of a reporto of our demonstration that the non-specific adenosine antagonist caffeine can in fact confer neuroprotection in a chonic dual pesticide toxin (parequat plus maneb) model of PD, of potentially greater environmental relevance than prior toxin models of PD.
- Demonstration and communcation of a protective effect of A_{2A} receptor depletion on chronic dopaminergic neuron toxicity induced by transgenic expression of mutant human alpha-synuclein.
- Using a powerful newly Cre/LoxP conditional knockout system, we have preliminarily reported evidence that neuronal forebrain A_{2A} receptors in the striatum contribute to this toxicity. Thus it is through these receptors that caffeine and more specific antagonists of the adenosine A_{2A} receptor may offer neuroprotection against the development or progression of PD.

Reportable Outcomes

1) Publications (with acknowledgements citing W81XWH-04-1-0881/ USAMRAA)

- Kachroo A, Richfield EK, Schwarzschild MA. 2008. Adenosine A2A receptor gene deletion confers protection in a transgenic alpha-synuclein model of Parkinson's disease. 2008 Neuroscience Meeting. Washington, DC: Society for Neuroscience. Abstract # 453.14/CC8. [See **Appendix B**.]

- Pisanu A, Sena-Esteves M, Schwarzschild MA. 2008. Effect of conditional knockout of striatal adenosine A2A receptors on MPTP-induced nigrostriatal toxicity in mice. 2008 Neuroscience Meeting. Washington, DC: Society for Neuroscience. Abstract # 453.6/BB34. [See **Appendix C**.]

Conclusions/Modifications/Plans

Central hypothesis: Multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease. Our progress under this award supports the central hypothesis, particularly with respect to caffeine-estrogen interactions in models of PD. In Year 4 we have obtained and reported evidence that the adenosine receptor blocker caffeine as well as specific genetic depletion of the A_{2A} subtype of adenosine receptor are capable of conferring neuroprotection in chronic pesticide and genetic mouse models of PD. Critical to our ability to successfully pursue the Specific Aims of our research program, we have made technical progress in assessing modification of a key conditioanl KO methodology developed in this project. We have presented initial data applying this method to the MPTP model of neurodegeneration in PD. Pursuit of the original aims to understand how caffeine and more specific A_{2A} receptor antagonists may offer a novel strategy to protecting neurons lost in Parkinson's disease is planned through a modification of the project approved this year, and to be completed in the next two years.

Plans: Approved modification (for Years 5 and 6) -- Abstract

A modification to the statement of work on our research project award W81XWH-04-1-0881, "*Caffeine Adenosine Receptors and Estrogen in Toxin Models of Parkinson's Disease*" is proposed in order to accomplish the original specific aims by incorporating new knowledge gained during the course of the original award. Specifically, we propose to build on our progress in pursuit of our original Specific Aims (SA) #1 (Experiment #2/3) and SA #2 (Experiments #5). Under SA #1 we will test the hypothesis that ***caffeine acts through blockade of either neuronal or astrocytic A_{2A}Rs in brain to protect dopaminergic neurons in both acute and chronic toxin models of Parkinosn's disease*** (PD). Based on a new appreciation that adenosine A_{2A} receptors expressed on non-neuronal cells may also play a key role in neuroprotection, we have modified the design of our originally proposed assessment of caffeine's neuroprotective effect in a chronic pesticide model of PD. We will determine if this protective effect is attenuated by the genetic depletion of A_{2A} receptors from either forebrain neurons or from astrocytes using an incisive set of recently characterized conditional knockout mice lacking either neuronal or glial A_{2A} receptors. Under SA #2 we will improve our test of the hypothesis that ***focal A_{2A} receptor inactivation in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity***. Based on refinements in our application of a viral *cre*-based conditional knockout system developed under the original award, we will determine whether anatomically restricted elimination of A_{2A} receptor in either the striatum or the substantia nigra can attenuate toxin-induced injury to dopaminergic nigro-striatal neurons. Having successfully achieved elements of SAs #1 and #2 and all of SA#3 during course of the original award, the proposed modification will afford achievement of all aims of W81XWH-04-1-0881.

Relevance: An improved understanding of how caffeine protects against toxin-induced dopaminergic neuron degeneration may be of substantial significance for the pathophysiology, epidemiology and therapy of PD. With specific adenosine A_{2A} antagonists emerging as a promising symptomatic treatment for PD, the prospects for their additional neuroprotective benefit as substantiated by this project may considerably enhance their therapeutic potential. Finally, the proposed experiments may have a broader impact in providing a prototype for modeling how environmental toxins and protectants as well as gender interact to influence neurological disorders associated with military and civilian exposures.

Rationale, Experimental Method and Intrpretation (for Mod. SA's M1 and M2)

Introduction to Modification: During the course of the funded project we have made substantial progress toward our original aims, as reflected in the details of our first 3 progress reports (attached as Appendices B through D), and the publications listed in each. Examples that highlight the contributions and impact of the project include our demonstration (under the original SA#1) that chronic repeated administration of caffeine, a non-specific adenosine receptor antagonist, can attenuate the dopaminergic neuron degeneration induced by pesticides (paraquat and maneb) in an environmentally relevant model of Parkinson's disease (PD); see Appendix A. In addition to their epidemiological and potentially therapeutic significance, these findings help establish a broad prototype for modeling the interplay of environmental factors that may both negatively (e.g., pesticides) and positively (e.g., caffeine) interact to influence the risk of neurodegenerative disease (e.g., PD). Similarly, our now published[1] demonstration (under the original SA#3) that estrogen modifies the protective effects of caffeine in the mouse MPTP model of PD remarkably parallels the interactions between estrogenic post-menopausal hormone use and caffeine use in their associations with the risk of PD, providing a major insight into the epidemiology of the disease.

Over the same period, the potential impact of this award on therapeutics for PD has been enhanced by progress in clinical development of adenosine antagonists for the treatment of PD. At least six biopharmaceutical companies are currently developing A_{2A} antagonists for PD, and at least four of these have advanced molecules to clinical studies.[2,3] Thus, the NIH clinical trial registry lists over a dozen actively recruiting or completed trials of novel A_{2A} antagonists for PD with independent sponsors (as identified by searching www.ClinicalTrials.gov for Kyowa's "KW-6002", Schering-Plough's "SCH 420814", or Biogen-Idec's "BIIB014"). In addition, a fourth industry program recently announced the advance of its A_{2A} antagonist Lu AA47070 into clinical trials,[4] and multiple other preclinical programs are apparently progressing (including publicly announced programs at Neurocrine[5] and Pharmacopeia[6]). We have provided evidence that adenosine A_{2A} receptors are required for caffeine's action as a dopaminergic neuron protectant (as demonstrated in part with the support of this award; see Fig. 1 of Appendix C/pg 7). Based on such findings, these emerging anti-parkinsonian adenosine antagonists are considered even more promising as they may possess disease-modifying as well as symptomatic benefits for PD patients.[2,15]

Despite the project's progress and impact, technical confounds (e.g., unexpected delayed toxic effects of the virus-mediated *cre* expression in the conditional A_{2A} knockout system we developed; see below) have left a few of the original key experiments incompletely pursued. This proposed modification of award W81XWH-04-1-0881 is designed to meet the original goals by completing or better accomplishing the most informative experiments – now with the benefit of new knowledge gained over the course of the funded project, as detailed below. ***The modified specific aims (SA's M1 and M2) proposed here are thus intended to strengthen the work and of original award, and help ensure its translational significance.***

Specific Aim M1: **to definitively determine whether brain A_{2A}Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD.**

Hypothesis M1: *Caffeine acts through blockade of brain A_{2A} receptors to protect dopaminergic neurons in chronic pesticides (paraquat/maneb) model of PD.*

Rationale:

The chronic paraquat/maneb (Pq/Mb) dual pesticide model of PD, i.e., chronic (twice weekly) exposure to these two commonly used pesticides, reproduces the core anatomical and biochemical features of PD (e.g., selective death of nigrostriatal neurons and loss of dopamine).[7,8] Since the award of this proposal, we have not only validated the Pq/Mb model in our laboratory, but also demonstrated that pretreatment with caffeine provided significant protection against tyrosine hydroxylase (TH)-positive neuronal cell loss induced by the pesticides. (Appendix A.)[9] These data support the neuroprotective role of caffeine in this environmentally relevant model of PD, though the mechanism of its protection is unclear. In another neurotoxin (MPTP) model of PD, we and others have shown that not only caffeine (a non-selective adenosine receptor antagonist) but also specific adenosine A_{2A}R antagonists protect against dopaminergic neurodegeneration. Moreover, we found that caffeine's neuroprotection against MPTP is lost in global A_{2A} KO mice, suggesting an indispensable role of A_{2A} in its protection.[10] However, the exact location of A_{2A}R responsible for this neuroprotection is still not clear. Studies in the MPTP model have so far provided inconsistent results, partially depending on different treatment paradigms. For example, it was reported recently that A_{2A}R activity in forebrain neurons was essential to the control of motor activity but not for protection against *acute* MPTP toxicity.[11] On the other hand, subchronic MPTP treatment-induced dopamine neuron death and glial activation were significantly attenuated in both A_{2A}R global KO mice and forebrain neuron-specific A_{2A}R KO mice, suggesting the importance of A_{2A}R particularly those in forebrain neurons for the protection against MPTP toxicity[12] (and unpublished results). Adenosine A_{2A}R antagonists are currently in clinical trials for PD because of their symptom-improving abilities. The neuroprotective properties of A_{2A}R antagonists significantly enhance the therapeutic potential of these agents for PD. As a first step in definitively localizing the adenosine receptor involved in the dopaminergic neuron toxicity, we will take advantage of a refined genetic knockout method that specifically eliminates A_{2A}Rs from the adult brain. By assessing caffeine's protective action in the presence and absence of brain A_{2A}Rs we will conclusively address the role and location of the A_{2A}R.

Technical background: We have developed two brain-specific conditional A_{2A} KO mice line based on a "Cre-Lox" recombination strategy.[13] (See more details of this methodology in original proposal) In these experiments we introduce the Cre gene by mating floxed A_{2A} mice with a transgenic line expressing the Cre recombinase. The two *cre* transgenes adopted for our study of brain A_{2A}R function are expressed under the control of the calcium/calmodulin-dependent protein kinase IIα gene (*CaMKIIα*) promotor or glial fibrillary acidic protein gene (*GFAP*) promotor, which drive expression primarily in the mouse forebrain neurons and astrocytes, respectively.

Since the award of this proposal we have obtained clear validation of this conditional knockout strategy with the characterization of homozygous floxed A_{2A}

littermates expressing the *cre* transgene and their non-transgenic littermates. Tissue-specific disruption of the forebrain neuronal $A_{2A}R$ was confirmed by PCR and western blot.[14] Functional studies have shown that caffeine-stimulated locomotion was lost in mice that are lacking $A_{2A}R$ only in forebrain neurons but not in those astrocytes, confirming the absence or presence of $A_{2A}R$ in striatum, respectively (Xu et al., 2006).

Experiment #M1a: **Effect of caffeine in Pq/Mb model using forebrain-neuron specific A_{2A} KO mice**.

Experimental design: Homozygous floxed $A_{2A}R$ littermates with or without *CAMKIIα-cre* (from matings of homozygous floxed A_{2A} mice, one with this *cre* tranagene and the other without) will be treated chronically with caffeine (20 mg/kg ip) or saline 10 min before Pq/Mb (as per protocol in Appendix A).[7,8] Paraquat (10 mg/kg i.p.) immediately followed by maneb (30 mg/kg i.p.) or (saline i.p. followed by saline i.p.) will be administered twice weekly for 8 weeks (n=8 for saline control group and n=16 for pesticides treated groups). Dopaminergic neuron degeneration will be measured one week after the last treatment by stereological cell counts of tyrosine hydroxylase-immunoreactive (TH-IR) cells in the substantia nigra using our established techniques (App. A). Parallel assessment of striatal dopaminergic neuron injury will be assessed by ^{3}H-mazindol autoradiography for the striatal DAT binding. Glia (astrocytes and microglia) activation will also be measured using immunohistochemistry.

Prediction and Interpretation: Based on our prior pharmacological and genetic studies, we predict that a robust caffeine protection of dopaminergic neurons in non-transgenic controls that will be completely lost in Cre-expressing conditional A_{2A} KO mice. This result would provide the strongest, most direct evidence to date that caffeine can protect dopaminergic neurons by acting on A_{2A}Rs that reside within the forebrain neurons (in a region that would be further localized in SA#2) rather than on A_{2A}Rs that are expressed in any of a number of peripheral tissues. On the other hand, the presence of caffeine's neuroprotection in the forebrain A_{2A} KO mice would suggest that caffeine works through A_{2A}Rs other than those in the forebrain neurons to protect against pesticides-induced neurotoxicity. This latter possibility will be explored in Exp.#2 in which caffeine's neuroprotection will be studied in mice that lack A_{2A}Rs in astrocytes.

Pitfalls and Alternatives: A potential pitfall of this experiment is that an unexpectedly pronounced protective phenotype of the brain-specific A_{2A} KO itself (>50% attenuation of the dopaminergic lesion) could mask a persistent protective effect of caffeine in the absence of brain A_{2A}Rs. In other words, the predicted lack of neuroprotection by caffeine in the KO mice could be due to caffeine's requirement for the $A_{2A}R$ *or* to a ceiling effect in which dopaminergic neurons are already largely protected. In the event that caffeine has no effect in brain-specific KO mice that themselves show near complete protection, then the experiment would be repeated and expanded to include a positive control for protection with a known $A_{2A}R$-

independent dopaminergic neuron protectant. If the brain-specific A_{2A} KO mice could be further protected by this agent but not by caffeine it would indicate that caffeine is in fact dependent on the A_{2A}R for this action.

Experiment #M1b: **Effect of caffeine in Pq/Mb model using astrocyte-directed A_{2A} KO mice**.

Experimental design: Homozygous floxed A_{2A}R littermates with or without *GFAP-cre* (from matings of homozygous floxed A_{2A} mice, one with this *cre* transgene and the other without) will be treated with caffeine or saline followed by Pq/Mb (or saline/saline) injections biweekly for 8 weeks as in Exp #1. Genotyping and assessments of dopaminergic neuron injury and degeneration will be conducted as above.

Prediction and Interpretation: Based on our prior experience using this line of mice in MPTP models (both single and subchronic injection paradigms), we predict that caffeine protection of dopaminergic neurons will be present in mice both with and without *GFAP-cre*. This result, together with the hypothesized result from Exp.#1 showing the lack of caffeine's effect in forebrain neuron specific A_{2A} KO mice, would provide further evidence that caffeine protects dopaminergic neurons by acting on A_{2A}Rs that reside within the forebrain neurons rather than on A_{2A}Rs that are expressed in non-neuronal brain tissues such as astrocytes. On the other hand, the loss of caffeine's neuroprotection in the *GFAP-cre* A_{2A} KO mice, together with the presence of caffeine's effect in *CAMKIIα-cre* A_{2A} KO mice, would suggest that caffeine depends on A_{2A}Rs in astrocytes rather than neurons to protect against pesticide-induced neurotoxicity.

Pitfalls and Alternatives: Although the primary target of Cre-mediated deletion directed by GFAP-cre mice was astroglia, various levels of Cre-mediated target gene deletion were also reported within some neuronal populations (e.g., hippocampus) in several independently generated *GFAP-cre* transgenic mouse lines, including the line used in this report. Therefore, the positive results from this experiment (i.e., the loss of neuroprotection by caffeine in *GFAP-cre* A_{2A} KO mice) could not totally exclude the contribution of A_{2A}R in other neurons for its protection. However, this Cre-mediated deletion has not been reported in nigrostriatal dopaminergic system. Moreover, the interpretation of this Exp. M1b with an astrocyte-directed A_{2A} KO line in the context of M1a (i.e., using the complementary neuron-directed conditional A_{2A} KO line) markedy reduces the risk of misinterpretation.

Specific Aim M2: **to determine whether focal depletion of adenosine A_{2A} receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity.**

Hypothesis M2: *Elimination of A_{2A} receptors in striatum (but not substantia nigra or cortex) using a viral* cre/loxP *conditional knockout system will protect dopaminergic nigrostriatal neurons from MPTP toxicity.*

Rationale: Microinjected AAV-*cre* offers a powerful method for testing hypothesis M2 by genetically inactivating the A_{2A} receptor in homozygous floxed *A_{2A}* mice with high spatial resolution at specific candidate sites. Although SA M1 is designed to localize the cellular source of A_{2A} receptor (to neuronal versus glial A_{2A} receptors), it does not address the regional localization of these receptors within brain. As reviewed in the original proposal caffeine acting on A_{2A} receptors expressed at any of several locations within the brain may be able to protect the dopaminergic neurons that degenerate in PD. The strongest arguments for ("*pro*") and against ("*con*") a contributing role of A_{2A} receptors at three specific sites in the demise of dopaminergic neurons:

Brain site	*pro*	*con*
1) striatum	High A_{2A}R expression on striato-pallidal neurons; prelim. data: App. D/pg11	Require retrograde or complex circuitry influence on nigra neurons (ie, indirect)
2) cortex	A_{2A}R's on cortico-striatal nerve terminals can facilitate excitotoxicity	Low density of A_{2A}R's on these neurons; also indirect
3) s. nigra	Any effect would be direct as it is nigral neurons that degenerate in PD	Extremely low levels of A_{2A}R's on these neurons

Technical background: A major focus of the parent project has been the development of the technical background needed to achieve SA M2. Although unexpected technical difficulty was encountered and delayed the key MPTP toxicity experiments originally proposed, substantial progress in characterizing the methodology and addressing the technical confounds has been made. Please see Appendices B-D, extensively detailing AAV-*cre* time course and dose-dependence, including that of main confound of local toxicity at the highest virus doses and latest time points (at 60 days post-AAV-*cre* infection; Appendix D/pg. 8). The technique was optimized further for elimination of A_{2A} receptors in the relatively large volume of the striatum by adding a second microinjection to cover both the rostral and caudal reaches of the striatum. Of note, the substantial yet discrete unilateral elimination of striatal A_{2A} receptors (e.g., Appendix B/pg 10, Appendix C/pg 12; Apendix D/pg 9) correlated with striking behavioral data showing mouse turning in response to adenosinergic and dopaminergic drugs in the pattern consistent with the asymmetric loss of the A_{2A} receptors' motor actions. (See Fig. 4, Appendix D/pg 10.)

The toxicological confounds noted 60d after AAV-*cre* infection notwithstanding, we were able to gather preliminary data in the MPTP model of PD suggesting that striatal A_{2A} receptors do indeed play an important role in MPTP-induced dopaminergic neuron injury. (Appendix D/pg 11.) Based on the time course data demonstrating stable maximal transgenic *cre* expression and A_{2A} receptor elimination one month (32 days) after AAV-*cre* infection of the striatum. (See panels A and C of Fig. 5 in Appendix C/pg 13.) Accordingly, we are now poised to conduct the full set of targeted regional A_{2A} receptor depletions (as originally proposed) now using a modified technique with earlier MPTP administration – one month after microinjections of AAV-*cre* (now double -- both rostral and caudal -- in striatum *vs* cortex *vs* substantia nigra) in floxed *A_{2A}* mice. As before non-floxed (i.e.,

wild type) mice and floxed A_{2A} mice infected instead with a control vector (AAV-*GFP*) will service as key controls.

Experiment M2: Design, Prediction and Interpretation, Pitfalls and Alternatives, and Justification of Animals: [As in original proposal.]

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Caffeine protects against combined paraquat and maneb-induced dopaminergic neuron degeneration

Anil Kachroo[1], Kavita Prasad[2], Michael C. Irrizary[1,Ψ], Eric K. Richfield[2], Michael A. Schwarzschild[1]*

[1]MassGeneral Institute for Neurodegenerative Disease, Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129; [2]Environmental and Occupational Health Sciences Institute, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 07920

[Ψ]Current address: GlaxoSmithKline, Worldwide Epidemiology, Research Triangle Park, NC 27709

[§]Corresponding author: Michael A. Schwarzschild, MassGeneral Institute for Neurodegenerative Disease, MGH, 114 Street, Charlestown, MA 02129, Telephone: 617-724-9611, Fax: 617-724-1480, email: michaels@helix.mgh.harvard.edu

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Abstract

Environmental exposures can be potentially neurotoxic as in the case of pesticides, which have been linked to an increased risk of Parkinson's disease (PD). Conversely, potentially protective factors such as the adenosine antagonist caffeine have been linked to a reduced risk of the disease. Caffeine and more specific adenosine A_{2A} receptor antagonists also consistently reduce dopaminergic neuron injury produced by acute toxin administration in animal models of PD. To determine whether caffeine confers also protection against chronic and possibly more environmentally relevant toxin exposures, its effects were investigated on dopaminergic neuron degeneration induced by eight weeks of combined treatment with the herbicide paraquat (PQ) and the fungicide maneb (MB) in mice. PQ (10mg/kg ip) plus MB (30mg/kg ip) were administered intraperitonally twice weekly in the absence or presence of caffeine. The number of nigral neurons that were positive for tyrosine hydroxylase immunoreactivity (TH-positive) was assessed using stereological methods and found to be significantly reduced (40%) by PQ and MB treatment. Neither caffeine nor combined PQ and MB treatment altered non-dopaminergic (TH-negative) nigral neuron counts. Caffeine at 20 mg/kg provided significant protection against TH-positive neuron loss, demonstrating that caffeine can protect against dopaminergic neuron degeneration induced by chronic exposure to pesticides in a mouse model of PD.

Parkinson's disease (PD) is a chronic neurodegenerative condition with cardinal features of rest tremor, rigidity and bradykinesia, which are attributed to an underlying neurodegeneration of dopaminergic neurons of the substantia nigra. Considerable evidence suggests an etiology for PD that includes both genetic and environmental factors. Non-genetic factors appear to be prominent in the majority of PD patients, in whom typical symptoms develop after age 50. Twin studies [1, 2] have generally identified low concordance rates for PD diagnosed after age 50 that did not differ by zygosity.

Amongst environmental factors, exposure to pesticides has been linked to an increase in the risk of PD. In addition to case-control studies, several prospective epidemiological investigations have supported an elevated risk of developing PD amongst those exposed to pesticides. Amongst Hawaiian men of Japanese ancestry the relative risk of developing PD tended to be increased (though did not reach statistical significance) for those individuals exposed to pesticides [3]. In an investigation of elderly subjects residing in France a significant increase in the relative risk of PD among men, but not women, was associated with occupational pesticide exposure [4].

Recently, a large prospective epidemiological study [5] of both men and women in the US found that current or regular past exposure to pesticides is associated with an increased risk for developing PD. Pesticide use was associated with a 70% increased risk of developing PD amongst men and women whose pesticide exposure history was reported prior to diagnosis. Though this study addressed the concern of recall bias that limited interpretation of prior studies, it did not identify specific pesticides as potential

environmental risk factors for PD. While most studies have shown a positive association between pesticide exposure and PD, no specific single agent has been implicated consistently. The herbicide paraquat (PQ) has emerged as a putative risk factor in part due to its structural similarity to 1-methyl-4-phenylpyridinium (MPP+), the active toxin metabolite of MPTP. In addition, occupational exposure to PQ has been associated with parkinsonism [6, 7]. However, common exposures to pesticides such as PQ may occur in combination with other pesticides, as single agents are often applied in overlapping geographical areas.[11] For example, diethyldithiocarbamates of which maneb (MB) is a member, are heavily used alongside PQ in certain parts of the US [8]. Occupational exposure to MB has been anecdotally linked to cases of parkinsonism in humans [9, 10].

Thiruchelvam *et al*. [11, 12] established a dual pesticide model of environmental parkinsonism in mice based on repeated systemic exposure to a combination of PQ and MB. Co-administration of these toxins was shown to produce selective lesions of nigrostriatal dopaminergic neurons, including loss of dopamine in the striatum and death of dopaminergic neurons in the substantia nigra. In addition to its potential environmental significance, this ‘dual pesticide’ mouse model entails exposure to PQ and MB over months and thus parallels the chronicity of putative environmental toxin exposure in humans, further enhancing its utility in the characterization of promising neuroprotective candidates for PD.

By contrast to pesticide exposure, the consumption of coffee and other caffeinated beverages has been repeatedly linked to a *reduced* risk of developing PD [13]. Caffeine itself appears to be the component that accounts for the association given that caffeinated,

but not decaf coffee was found to be associated with a lower PD risk in a large prospectively followed population [14].

Laboratory studies from our and other groups have complemented these epidemiological findings to suggest that the inverse association between caffeine and PD risk may be due to a neuroprotective action of caffeine. We found caffeine at a dose in mice corresponding to as little as a single cup of coffee in humans could significantly attenuate the loss of striatal dopamine induced by acute exposure to the dopaminergic neuron toxin MPTP [15]. Similarly, caffeine can reduce nigrostriatal neuron injury triggered by a single dose of locally administered 6-OHDA in rats. [16, 17]. The biological plausibility of a neuroprotective action of caffeine in PD was strengthened with the repeated demonstration that antagonists of the adenosine A_{2A} (but not A_1) receptor mimic the effects of caffeine in these acute MPTP and 6-OHDA toxin models of PD [15, 18, 19, 20]. Genetic disruption of the A_{2A} receptor similarly attenuated acute neurotoxicity in the MPTP mouse model of PD [15].

These convergent epidemiological and laboratory data have prompted the inclusion of caffeine on a short list of candidate neuroprotectants warranting consideration for disease-modifying therapy in clinical neuroprotection trials for PD [21]. However, the recent failure of several clinical neuroprotection trials of agents that had emerged as promising candidate neuroprotectants from the preclinical pipeline for PD has prompted a reexamination of the animal models supporting the pipeline as well as the clinical trial methodology used to test the candidates [22, 23]. These concerns have encouraged the development and use of additional animal models of PD neurodegeneration (beyond the traditional acute toxin models), with particular emphasis

on models that are more intuitively relevant to PD, for example, because they better mimic the pathology or chronicity of the disease [24, 25]. Accordingly, the current study seeks to better assess the neuroprotective potential of caffeine by testing its ability to alter the dopaminergic nigral neuron degeneration induced by chronic exposure to pesticides [11, 12].

Two-month-old male C57BL/6 mice were obtained from Charles River Laboratories; Wilmington, MA and housed under a 12:12 hr light:dark cycle. Food and water was provided *ad libitum.* All experiments were performed in accordance with Massachusetts General Hospital and NIH guidelines on the ethical use of animals, with adequate measures taken to minimize pain and discomfort. Mice were injected intraperitoneally with saline (vehicle), caffeine 5mg/kg or caffeine 20 mg/kg (Sigma), followed 10 minutes later by a pair of intraperitoneal (i.p.) injections, either saline for both or 10 mg/kg PQ (1,1'-dimethyl-4,4'-bipyridinium) dichloride hydrate (Sigma) first and 30 mg/kg MB (manganese bisethylenedithiocarbamate) (Chem Service) second. PQ and MB were dissolved separately in saline on the day of administration. Mice were treated chronically (twice weekly for 8 weeks) in the following randomly assigned groups: Saline control, (n=8); PQ and MB, (n=12); Caffeine (5mg/kg) control, (n=8); Caffeine (5mg/kg) + (PQ and MB), (n=12); Caffeine (20mg/kg) control, (n=8); and Caffeine (20mg/kg) + (PQ and MB), (n=12). A mortality rate of 42% in the toxin-treated groups (consistent with prior experience in this PQ and MB paradigm; M. Thiruchelvam, personal communication) occurred equally (5 of 12 mice) across these three groups, and thus is unlikely to affect comparisons between them. Body weights were obtained twice a week during the course of the experiment. No changes in body weight were produced by

any of the treatments, consistent with previous observations [11]. Animals were sacrificed one week after the last injection.

During the experimental paradigm horizontal locomotor activity, subdivided into ambulation and fine movement, was assessed by an automated recording system (San Diego Instruments) in standard polypropylene cages (15 x 25 cm) placed into frames equipped with 5 infrared photocell beams (5 cm apart). Ambulation was measured as the number of sequential breaks in 2 adjacent beams and fine motor activity (which can reflect grooming and other stereotyped activities) was measured as the number of sequential breaks in a single beam. Photobeam breaks were recorded in 10 min intervals for 3 hours. Mice were not tested on treatment days but 24-48 h later. Motor assessments were performed 4 times over the 8-week treatment period for a total of 4 sessions. No changes in motor activity were observed during the course of the experiment by any of the treatments (data not shown).

One week after the last toxin injection (week 9), mouse brains were removed and immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and stored at 4 °C overnight. Paraformaldehyde-fixed brains were then placed in 30% sucrose (cryoprotectant) until the next day. Brains were then rinsed in PBS, placed in aluminum foil and stored at -80°C. The fixed brains were then cut on a Leica microtome into 40-µm thick sections for immunolabelling studies. The free floating sections were washed three times in PBS and then treated with a 20:1 fold mixture of 3% hydrogen peroxide and Triton X-100. The sections were washed again three times in PBS before pre-incubation in a blocking solution of 3% milk for one hour. After a further three washes, sections were incubated overnight at 4ºC with a primary antibody to tyrosine hydroxylase

(monoclonal anti-TH; 1:800, Sigma), the rate-limiting enzyme in dopamine biosynthesis and a marker for dopaminergic neurons. The next day the sections were washed three more times before incubation with a secondary biotinylated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) at 1:200 for one hour. This step was followed by three washes and incubation for one hour in ABC solution (Vectastatin, Vector Labs), before the sections were further washed in PBS and then finally developed in 0.3 mg/ml 3-3'-diaminobenzidine tetrachloride (DAB). The stained free floating sections were then mounted on glass slides and cover-slipped. These slides were later counterstained with cresyl violet acetate (9-amino-5-imino-5H-benzo[a]phenoxazine acetate salt) obtained from Sigma for Nissl staining. This counterstaining procedure involved the slides being placed in fresh xylene (4 min) followed by ethanol at 100% (8 min), 95% (6 min), 70% (4 min) then water for 2 min. The slides were then immersed in 0.5% cresyl violet acetate solution for 20 min, followed by 2 quick dips in water, 70% ethanol (2min) and a differentiator solution (mixture of 125:1, 95% ethanol and 100% glacial acetic acid) for 1 min allowing rapid decolorization of the Nissl stain. Sections were then dehydrated by placing the slides in 95% ethanol (4 min) and 100% ethanol (4 min) and finally xylene (10 min) before mounting the cover slip.

Stereological assessment of neuronal loss in midbrain sections was limited to the substantia nigra pars compacta (SNpc) because chronic PQ and MB exposure had been previously shown to cause degeneration of dopaminergic neurons of the SNpc and not the adjacent (medial) ventral tegmental area [12]. After delineation of the SNpc at low magnification (10 x objectives) the entire region was sampled at higher magnification (40 x objectives). The total number of TH-positive and Nissl-positive only stained TH-

negative neurons in the SNpc were counted using an optical fractionator method and quantifications were carried out using Bioquant software. TH-negative neurons were counted to assess whether any reduction in the number of TH-positive neurons represent neuronal cell death *versus* the extent of TH expression or staining intensity. The investigator was blinded to the treatments to avoid bias during the counting process.

All values are expressed as Means ± SEM. Single statistical comparisons between respective control and treated groups were performed using an unpaired *t*-test; multiple group comparisons were performed using one-way ANOVA followed by *post hoc* analysis.

Combined PQ and MB treatments significantly decreased the number of TH-positive neurons in the SNpc by approximately 40% compared to the corresponding saline-treated group (Figure 1a; $p<0.005$, unpaired *t*-test). Prior caffeine treatments at 20 but not 5mg/kg provided a significant ($p<0.05$, one-way ANOVA followed by *post hoc* analysis) attenuation in the loss of TH-positive neurons, suggesting almost complete neuroprotection (compared to respective caffeine control group) against toxin-induced neuronal cell loss. Total Nissl-positive neurons (i.e., total number of neurons) showed a similar profile to TH-positive neuronal cell counts suggesting that these toxins only targeted the dopaminergic neurons of the SNpc (Figure 1c). Direct assessment of TH-negative neurons confirmed that there was no effect of the pesticides on this subpopulation of nigral neurons (Figure 1b; $p>0.05$, one-way ANOVA). Sub-analysis of the data confirmed that changes in total nigral neuron density were comparable to changes in TH-positive neuronal cell counts (Figure 2a). Assessment of the total volume

of the SNpc between groups showed no significant difference (Figure 2b; $p>0.05$, one way ANOVA).

This study sought to determine whether caffeine pretreatment would afford protection against dopaminergic neuron toxicity in mice chronically exposed to a combined PQ and MB. In this setting, prior caffeine exposure did in fact reduce the loss of TH-positive neurons triggered by pesticide treatment suggesting a neuroprotective effect. However, an alternative explanation is that caffeine up-regulated TH as indicated by the tendency toward an elevation of TH-positive neuronal cells in caffeine control groups compared to the saline control group. Chronic caffeine treatment at 20mg/kg has previously been shown to increase expression of TH in nigral neurons [26]. If caffeine were indeed up-regulating TH in our experiment, then the observed increase in TH-positive cell counts, albeit not significant, should correlate with a complementary decrease in TH-negative neurons. Such a conversion of nigral neuron phenotype appeared not to be the case, demonstrating that caffeine was neuroprotective in this study.

Locomotor activity was monitored throughout this experiment to provide a behavorial surrogate of pesticide toxicity. However, PQ and MB treatment failed to reduce basal motor activity after 8 weeks of toxin treatment. Thiruchelvam *et al.* [12] had administered PQ and MB twice a week for 6 weeks to 6-week-old mice and demonstrated a motor deficit 24 hours after the last injection (6 week) time-point. This discrepancy in between studies may be due to methodological differences. For example, the different substrains of C57Bl/6 mice employed in their study and ours may contribute to different functional manifestations of neurotoxin-induced injury to nigrostriatal dopaminergic neurons in C57Bl/6 substrains [27, 28].

The antiparkinsonian potential of A_{2A} antagonism has been bolstered by convergent epidemiological studies and laboratory data, showing that A_{2A} antagonists protect against acute toxin exposure in the MPTP and 6-OHDA models of PD [13]. Here we demonstrate that the neuroprotective potential of caffeine extends to a chronic and potentially more environmentally relevant 'dual pesticide' model of PD. These data also extend our preliminary findings in the MPTP model [29] that caffeine can prevent the degeneration *per se* of dopaminergic neurons, as well as their dysfunction. Establishing the ability of caffeine to protect dopaminergic neurons in this model further supports (but does not prove) the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with the risk of PD. The study also strengthens the rationale for consideration of caffeine and more specific A_{2A} antagonists as therapeutic tools for slowing the underlying degenerative process.

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Figure legends

FIGURE 1. Neuronal cell counts of the substantia nigra pars compacta. Animals were sacrificed 1 week after the last treatment of either saline or combination of 10mg/kg paraquat and 30mg/kg maneb either in the presence or absence of caffeine. (A) Total number of TH-positive neurons per (bilateral) substantia nigra. (B) Total number of TH-negative neurons (only cresyl violet-positive) per substantia nigra. (C) Total combined counts of TH-positive neurons and TH-negative per substantia nigra. Data are represented as group mean ± SEM. *$p<0.005$, **$p<0.001$ vs respective control, unpaired *t*-test; $^{\#}p<0.05$ vs saline + paraquat and maneb treatment; one-way ANOVA followed by *post-hoc* analysis).

FIGURE 2. Effect of drug treatment on neuronal cell density and nigral volume. (A) Total calculated density of TH-positive neurons in the substantia nigra pars compacta. (B) Total calculated volume of the substantia nigra pars compacta. Data are represented as group means $\pm$ SEM. (*$p<0.005$, **$p<0.05$ vs. respective control, unpaired t-test; $^{\#}p<0.05$ vs. caffeine (5mg/kg) + paraquat and maneb treatment; one-way ANOVA followed by *post-hoc* analysis).

Figure 1. Kachroo *et al.*

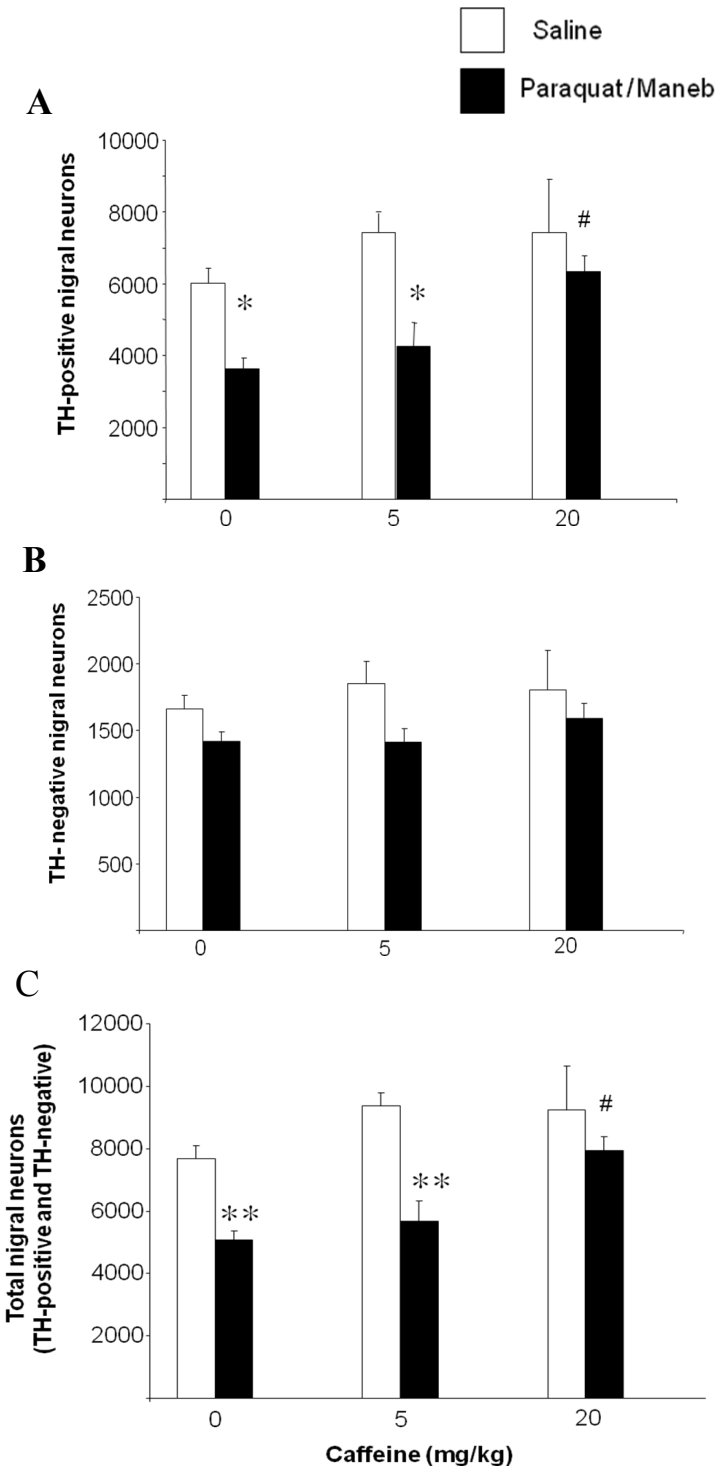
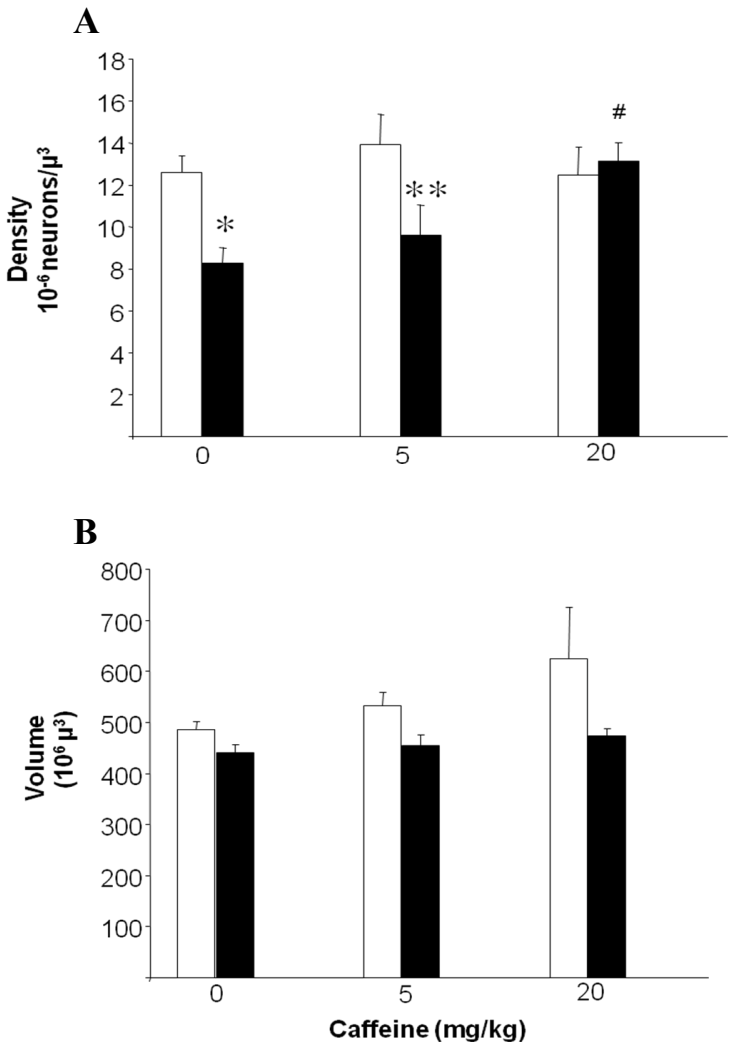


Figure 2. Kachroo *et al*.



Appendix B

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Presentation Abstract

Program#/Poster#:	453.14/CC8
Title:	Adenosine A_{2A} receptor gene deletion confers protection in a transgenic α-synuclein model of Parkinson's disease
Location:	Washington Convention Center: Hall A-C
Presentation Time:	Monday, Nov 17, 2008, 2:00 PM - 3:00 PM
Authors:	***A. KACHROO**[1], E. K. RICHFIELD[2], M. A. SCHWARZSCHILD[1]; [1]Dept of Neurol., Mass Gen. Hosp/Harvard Med. Sch., Charlestown, MA; [2]Envrn. and Occup. Hlth. Sci. Institute, Univ. of Med. and Dent. of New Jersey, Piscataway, NJ
Abstract:	Parkinson's disease (PD), a progressive neurodegenerative disorder, results from the interaction of genetic and environmental factors in association with aging. From a genetic standpoint human α-synuclein (*hw-αSYN*) has been implicated in the PD phenotype. Mutations in the human α-synuclein gene (A53T, A30P, 3rd one) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human α-synuclein gene with two of these mutations have been shown to develop age-related reductions in number of dopaminergic nigral neuron, level of striatal dopamine (DA) and locomotor activity. Pharmacological blockade of the adenosine A_{2A} receptor using caffeine (non-specific A_{2A} antagonist) and specific antagonists, as well as genetic elimination of the A_{2A} receptor in mice have all been shown to be neuroprotective in mouse models of PD. The present study sought to determine whether deletion of the A_{2A} receptor (A_{2A}[-/-] mice) would afford protection against the age-related reduction of striatal

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dopamine in double-mutant human α-synuclein (*hm^2-αSYN*) mice. Heterozygous *A_{2A}*[+/-] mice were mated with *A_{2A}*[+/-] mice that were also transgenic for the wild-type (*hw-αSYN*) or doubly mutated (*hm^2-αSYN)* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter - all in the C57Bl/6J background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional A_{2A} receptor, DA content was significantly lower (by 35%) in hm^2-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in *A_{2A}*[-/-] mice (lacking functional A_{2A} receptor) the hm^2-αSYN transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender. Thus the A_{2A} receptor appears to be required for neurotoxicity in this α-synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific A_{2A} antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

Disclosures: **A. Kachroo** , None; **E.K. Richfield**, None; **M.A. Schwarzschild**, None.

Support: NIEHS Grant R01010804

DOD Grant W81XWH-04-1-0881

AFAR/Beeson program

[Authors]. [Abstract Title]. Program No. XXX.XX. 2008 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2008. Online.

Appendix C

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Presentation Abstract

Program#/Poster#:	453.6/BB34
Title:	Effect of conditional knockout of striatal adenosine A2A receptors on MPTP-induced nigrostriatal toxicity in mice
Location:	Washington Convention Center: Hall A-C
Presentation Time:	Monday, Nov 17, 2008, 2:00 PM - 3:00 PM
Authors:	***A. PISANU**, M. SENA-ESTEVES, M. A. SCHWARZSCHILD; Neurol, Massachusetts Gen. Hosp., Charlestown, MA

Abstract: Over the past decade large prospective epidemiologic studies have linked consumption of coffee and other caffeinated beverages to a reduced risk of developing Parkinson's disease (PD) later in life, raising the possibility that caffeine might protect dopaminergic neurons from degeneration in PD. Experimental evidence has shown that caffeine, as well as more specific antagonists of adenosine A_{2A} receptors (A_{2A}Rs), attenuates neurotoxicity in experimental models of PD, and that MPTP-induced losses of striatal dopamine and dopamine transporters are significantly attenuated in A_{2A}R knockout (KO) mice compared to their wild-type littermate. But how and where A_{2A}R blockade or disruption influences the death of nigrostriatal dopaminergic neurons remains still uncertain. There is little evidence for appreciable expression of A_{2A}Rs on dopaminergic nigrostriatal neurons; whereas high levels are expressed on GABAergic striatopallidal output neurons, which are postsynaptic to the dopaminergic neurons that degenerate in PD. To better address the role of striatal A_{2A}R in neurotoxicity we took advantage of a viral Cre/*loxP* conditional KO system: infusion of an adeno-associated virus (AAV) vector encoding Cre

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recombinase into the striatum of homozygous floxed $A_{2A}R$ ($A_{2A}R^{flox/flox}$) adult mice induces a selective depletion of $A_{2A}R$ in infected cells. Using this AAV-*cre*/*loxP* system, a double injection of AAV2/1-*cre* ($3x10^{13}$ gc/ml) into the rostral and caudal striatum on one side induced a 50% loss of striatal $A_{2A}R$ expression by day 30 post-injection. This focal $A_{2A}R$ KO phenotype remained stable at day 60 post-injection, as revealed by immunohistochemical detection of *cre* expression and absence of $A_{2A}R$ expression in the same area. Fifty days after infection, AAV2/1-*cre* injected $A_{2A}R^{flox/flox}$ mice and their respective AAV2/1-*GFP* controls were treated with MPTP (15mg/kg) or saline i.p every 2 hours for a total of 4 doses. Striatal Tyrosine hydroxylase (TH) immunoreactivity (IR), representing dopaminergic projections, was measured one week later. In control AAV2/1-*GFP*-injected mice, the loss of striatal dopamine was indistinguishable between ipsilateral and contralateral striata. By contrast, in AAV2/1-*cre*-injected mice the intensity of TH IR was higher in injected than in contralateral striatum. Further stereological analysis of surviving neurons in substantia nigra *pars compacta* is in progress - in order to confirm whether focal disruption of $A_{2A}R$ in the striatum could confer neuroprotection against MPTP toxicity in this model of PD.

Disclosures: **A. Pisanu** , None; **M. Sena-Esteves**, None; **M.A. Schwarzschild**, None.

Support: DOD W81XWH-04-1-0881

NIEHS R01010804

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[Authors]. [Abstract Title]. Program No. XXX.XX. 2008 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2008. Online.